FOR INFORMATION
DA-2008-19
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SUBJECT: *Phytophthora ramorum* (ramorum blight, ramorum dieback, sudden oak death): New Real-time PCR System for Molecular Diagnosis of *P. ramorum*.

TO: STATE AND TERRITORY AGRICULTURAL REGULATORY OFFICIALS

The *P. ramorum* program currently uses a combination of two conventional PCR (Polymerase Chain Reaction) assays for molecular diagnosis of samples. These two conventional PCR assays (nested and multiplex) target the Internal Transcribed Sequence (ITS), specifically ITS-2 and ITS-1 & -2 regions respectively, and provide a quality control reaction to assure extracted DNA is of sufficient quantity and quality for analysis. The APHIS regulatory program has used this combined assay since 2004. In addition, a Real-time PCR assay that also targets the ITS-1 region and has a plant DNA internal control was approved and added for diagnostic determinations starting in 2006.

Review by the National Plant Board and other stakeholders of the diagnostic assays used for the *P. ramorum* program produced recommendations that CPHST (Center for Plant Health, Science and Technology) work to improve assays: by moving to Real-time PCR formats, by requiring that confirmation be made using at least two separate genomic targets in the causal agent, and by using target and control reactions that have enhanced specificity thereby reducing the risk of false positives caused by cross reaction of related species or through contamination of samples.

The CPHST National Plant Germplasm and Biotechnology Laboratory in Beltsville, Maryland, has responded to this request by evaluating five Real-time PCR assays that have become available within the past few years: Elicitin with a 5.8S *Phytophthora* internal control multiplex assay (developed by the Canadian Food Inspection Agency - CFIA), COX with a plant DNA internal control multiplex assay (developed by the USDA Agricultural Research Service), ß-Tubulin and ITS multiplex assays both without an internal control (developed by CFIA) and the *Ypt1* assay without an internal control (developed by the Scottish Crop Research Institute, Dundee, Scotland). A comparative analysis was performed on the following parameters: sensitivity, specificity and the potential for generating false positives and false negatives.

For a participating laboratory wishing to switch their current diagnostics exclusively to Real-time PCR formats, we are recommending combining the current ITS Real-time PCR assay with the addition of the CFIA Elicitin/5.8S internal control Real-time assay. We do not recommend that either assay be used without the other for making regulatory determinations. The basis of this recommendation is that it provides;
1) The sensitivity of the current ITS based Real-time PCR which is greater than other current Real-time PCR assays tested,
2) The demonstrated specificity of the Elicitin/5.8S Real-time PCR assay,
3) The separateness of the genomic targets used by both assays, and
4) Two reciprocal control reactions provided by the combined use of the Real-time PCR assays which parallel the control reactions in the current diagnostic protocols.

In some instances, other assays evaluated in this study provided comparable sensitivity and specificity to the recommended CFIA Elicitin assay. However, use of the existing ITS Real-time PCR assay with the Elicitin/5.8S assay combine features that best address stakeholder recommendations and program needs.

Please note that the approved conventional PCR assays will need to continue to be used to diagnose low concentration samples if the paired ITS and Elicitin/5.8S Real-time PCR assays produce an inconclusive result. We anticipate that paired ITS and Elicitin/5.8S Real-time PCR assays will rarely produce an inconclusive result. In addition, we continue to recommend the combination of the current conventional and current Real-time PCR assays as an optional assay, if requested, since the nested conventional PCR assay continues to be the most sensitive PCR assay we have evaluated to date. This option will also remain available for laboratories that do not possess Real-time PCR capabilities but wish to continue as a laboratory approved for *P. ramorum* molecular diagnostics.

The APHIS *P. ramorum* Program expects the new diagnostic protocols to be in place for use on regulatory samples by the 2009 testing season. To accomplish this goal, the roll-out of this new Real-time PCR assay will occur in stages. The new protocol will be transferred for immediate implementation to the PPQ-PHP- Molecular Diagnostic Laboratory (MDL) for use in PPQ confirmatory diagnostic procedures during May, 2008. By June, the protocol will be released to laboratories participating in the developing NPPLAP (National Plant Protection Laboratory Accreditation Program) that have Analysts performing *P. ramorum* diagnostics on behalf of PPQ, and to State, University, and NPDN (National Plant Diagnostic Network) laboratories. The purpose of this second release is so that Analysts participating in NPPLAP can prepare for the 2009 season by obtaining reagents and gaining hands-on experience in the use of the assay in test control reactions prior to implementation by the APHIS *P. ramorum* Program. The protocol for this new PCR procedure will be posted to the APHIS *P. ramorum* website: http://www.aphis.usda.gov/plant_health/plant_pest_info/pram

For more information on this or the Federal *P. ramorum* regulatory program, you may contact the National *P. ramorum* Program Manager, Jonathan Jones, at (301) 734-5038.

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